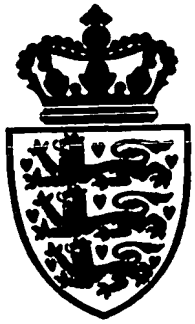
PRIORITY DOCUMENT
SUBMITTED OR TRANSMITTED IN COMPLIANCE WITH RULE 17.1(a) OR (b)



Kongeriget Danmark

EP/04/51280

Patent application No.: PA 2003 01067

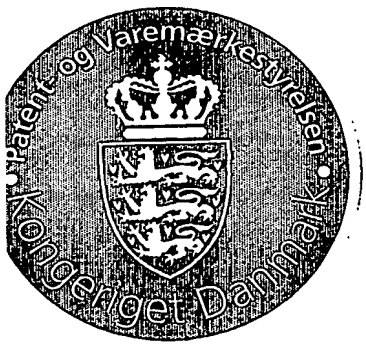
Date of filing: 11 July 2003

Applicant: (Name and address)
ZGene A/S
Anker Engelundsvej 1, Bygning 301
DK-2800 Lyngby
Denmark

Titlel: Yellow fever mosquito deoxyribonucleoside kinase and its use

IPC:

This is to certify that the attached documents are exact copies of the above mentioned patent application as originally filed.



Patent- og Varemærkestyrelsen
Økonomi- og Erhvervsministeriet

29 June 2004

Pia Høybye-Olsen

PATENT- OG VAREMÆRKESTYRELSEN

11 JULI 2003
Modtaget

YELLOW FEVER MOSQUITO DEOXYRIBONUCLEOSIDE KINASE AND ITS USE

TECHNICAL FIELD

This invention relates to a gene encoding mosquito multisubstrate deoxyribonucleoside kinase (dNK) and its use in nucleoside analogs activation and gene tharapy. In particular the invention relates to novel deoxyribonucleoside kinases derived from yellow fever mosquito *Aedes aegypti*.

In further aspects the invention provides novel gene and polynucleotide encoding the deoxyribonucleoside kinases, vector and recombinant virus constructs comprising the said gene, host cells carrying the polynucleotide or vector, methods of sensitising cells to prodrugs, method of inhibiting unvanted cell growth in warm-blooded animals, methods of synthesizing monophosphates, imagining applications and pharmaceutical compositions comprising the deoxyribonucleoside kinases of the invention.

In a preferred embodiment the invention provides a unique combination of a mosquito dNK kinase and the nucleoside analog gemcitabine to treat abnormal cell growth.

BACKGROUND ART

DNA is made of four deoxyribonucleoside triphosphates, provided by the *de novo* and the salvage pathway. The key enzyme of the *de novo* pathway is ribonucleotide reductase, which catalyses the reduction of the 2'-OH group of the nucleoside diphosphates, and the key salvage enzymes are the deoxyribonucleoside kinases, which phosphorylate deoxyribonucleosides to the corresponding deoxyribonucleoside monophosphates.

Deoxyribonucleoside kinases from various organisms differ in their substrate specificity, regulation of gene expression and cellular localisation. In mammalian cells there are four enzymes with overlapping specificities, the thymidine kinases 1 (TK1) and 2 (TK2), deoxycytidine kinase (dCK) and deoxyguanosine kinase (dGK) phosphorylate purine and pyrimidine deoxyribonucleosides. TK1 and TK2, which are pyrimidine specific and phosphorylate deoxyuridine (dUrd) and thymidine (dThd), and TK2 also phosphorylates deoxycytidine (dCyd). dCK phosphorylates dCyd, deoxyadenosine (dAdo) and deoxyguanosine (dGuo), but not dThd. dGK phosphorylates dGuo and dAdo. TK1 is cytosolic, and TK2 and dGK are localised in the mitochondria, although recent reports indicate a cytoplasmic localisation of TK2 as well. The same enzymes are also responsible for converting nucleoside analogs to therapeutically active nucleotide forms.

Nucleoside analogs are widely used in treatmant of various cancer and viral diseases. The analogs are inactive prodrugs that are dependent on intracellular phosphorylation to fully exert theraputic effect. A prodrug activation strategy for selectively imparing tumor cells involves the expression of a gene encoding an exogeneous enzyme in the tumor cells and administration of a substrate for that enzyme. The enzyme acts on the substrate to generate a substance toxic to the targeted tumor cells.

Several patents disclose use of human *Herpes simplex* virus 1 thymidine kinase (HSV-TK1) for cancer gene therapy treatment. Thymidine kinase, expressed in tumor cells, converts nucleoside analog prodrugs, such as acyclovir (ACV) or gancyclovir (GCV), into active form which incorporate in DNA and consequently kill the tumor. The use of HSV-TK1 in combination with several other nucleoside analogs has been suggested. However, no experimental work towards an effective combination of gemcitabine and a thymidine kinase for use in the treatment of human cancer or in other human abnormal cell growth related diseases has been accomplished.

An EST from *Aedes aegypti* have been submitted in GenBank™ (Accession No. CB251541). However, to this date no anotation has been provided, no experimental work towards characterisation, properties, localisation, use or biological function of this partial gene has yet been accomplished. This partial sequence is not sufficient for expression of the active protein. The full sequence encoding for mosquito dNK was isolated, sequenced, characterized and shown to possess deoxyribonucleoside kinase activity.

SUMMARY OF THE INVENTION

It is an object of the present invention to provide mosquito dNK useful for converting nucleoside analogs into toxic substances, and useful for converting nucleosides into monophosphates.

More specifically the invention provides a unique combination of a mosquito dNK kinase and the nucleoside analog gemcitabine to treat abnormal cell growth.

In its second aspect the invention provides isolated polynucleotides encoding a dNK kinase enzyme derived from *Aedes aegypti.*

In a third aspect the invention provides expression vector constructs comprising the polynucleotide of the invention, and optionally a promoter operably linked to the polynucleotide.

In a fourth aspect the invention provides packaging cell lines capable of producing infective virions, which cell line comprises the expression vector of the invention.

In a fifth aspect the invention provides host cells carrying the polynucleotide of the invention, or the expression vector of the invention.

In a sixth aspect the invention provides pharmaceutical compositions comprising the mosquito dNK kinase enzyme of the invention, the expression vector of the invention, or the host cell of the invention, and a pharmaceutically acceptable carrier or diluent.

In a seventh aspect the invention provides method of sensitising target cells to prodrugs, which method comprises the steps of (i) transfecting said target cell with a polynucleotide sequence of the invention, which encodes an enzyme that promotes the conversion of said prodrug into a (cytotoxic) drug; and (ii) delivering said prodrug to said cell; wherein said cell is more sensitive to said (cytotoxic) drug than to said prodrug.

In an eight aspect the invention provides methods of inhibiting pathogenic agents in warm-blooded animals, which method comprises administering to said animal a polynucleotide of the invention, or a vector of the invention.

In a ninth aspect the invention relates to the use of the mosquito dNK kinase for radionucleotide imaging for biodistribution studies.

In a tenth aspect the invention relates to the use of the mosquito dNK kinase enzyme of the invention for the phosphorylation of a nucleoside or a nucleoside analog.

In a eleventh aspect the invention provides methods of phosphorylating a nucleoside or a nucleoside analog, comprising the steps of (i) subjecting the nucleoside or nucleoside analog to the action of the mosquito dNK kinase enzyme of the invention, and (ii) recovering the phosphorylated nucleoside or nucleoside analog.

Other objects of the invention will be apparent to the person skilled in the art from the following detailed description and examples.

DETAILED DISCLOSURE OF THE INVENTION

Yellow fever mosquito *Aedes aegypti* dNK Kinase

In its first aspect the invention provides novel protein having multisubstrate deoxyribonucleoside kinase (dNK) enzyme activity, and which protein is derived from mosquito. More specifically the novel dNK enzyme is derived from yellow fever mosquito *Aedes aegypti*.

The dNK kinase enzyme of the invention is particularly useful for the treatment of abnormal cell growth by activating nucleoside analogs, in particular gemcitabine.

Identity of Polypeptides

In another preferred embodiment the mosquito dNK enzyme of the invention comprises the amino acid sequence presented as SEQ ID NO: 2, or an amino acid sequence that has at least 30%, preferably at least 50%, even more preferred at least 70%, still more preferred at least 80%, yet more preferred at least 90%, even more preferred at least 95% identity, most preferred at least 98 % identity, when determined over its entire length.

In the context of this invention "identity" is a measure of the degree of homology of amino acid sequences. In order to characterize the identity, subject sequences are aligned so that the highest order homology (match) is obtained. Based on these general principles the "percent identity" of two amino acid sequences is determined using the BLASTP algorithm [*Tatiana A. Tatusova, Thomas L. Madden*: Blast 2 sequences - a new tool for comparing protein and nucleotide sequences; FEMS Microbiol. Lett. 1999 **174** 247-250], which is available from the National Center for Biotechnology Information (NCBI) web site, and using the default settings suggested here (i.e. Matrix = Blosum62; Open gap = 11; Extension gap = 1; Penalties gab x_dropoff = 50; Expect = 10; Word size = 3; Filter on).

The results of this BLASTP comparison are presented in Table 1.

Table 1

BLASTP Comparison of *Aedes aegypti* dNK Protein Sequence with dNKs of Different Insect Origin

Aedes dNK BLAST P	*D. melanogaster*	*B. mori*	*A. gamibiae*
Identities	159/248 (64%)	133/237 (56%)	191/244 (78%)
Positives	189/248 (76%)	166/237 (69%)	215/244 (87%)
Gaps	4/248 (1%)	13/237 (5%)	1/244 (0%)

Identities / Length of the compared fragment / Identities (%)

In a preferred embodiment the dNK enzyme of the invention is derived from *Aedes aegypti*, and comprises at least 60%, preferably at least 70%, more preferred at least 80%, even more preferred at least 90%, most preferred at least 95% of the amino acid residues presented in SEQ ID NO: 2.

Variant Polypeptides

In a most preferred embodiment the mosquito dNK enzyme of the invention comprises the amino acid sequence presented as SEQ ID NO: 2, or a functional analogue thereof.

In the context of this invention, the term "functional analog" means a polypeptide (or protein) having an amino acid sequence that differs from the sequence presented as as SEQ ID NO: 2, at one or more amino acid positions. Such analogous polypeptides include polypeptides comprising conservative substitutions, splice variants, isoforms, homologues from other species, and polymorphisms.

As defined herein, the term "conservative substitutions" denotes the replacement of an amino acid residue by another, biologically similar residue. Examples of conservative substitutions include

(i) the substitution of one non-polar or hydrophobic residue such as alanine, leucine, isoleucine, valine, proline, methionine, phenylalanine or tryptophan for another, in particular the substitution of alanine, leucine, isoleucine, valine or proline for another; or

(ii) the substitution of one neutral (uncharged) polar residue such as serine, threonine, tyrosine, asparagine, glutamine, or cysteine for another, in particular the substitution of arginine for lysine, glutamic for aspartic acid, or glutamine for asparagine; or

(iii) the substitution of a positively charged residue such as lysine, arginine or histidine for another; or

(iv) the substitution of a negatively charged residue such as aspartic acid or glutamic acid for another.

The term conservative substitution also include the use of a substituted amino acid residue in place of a parent amino acid residue, provided that antibodies raised to the substituted polypeptide also immunoreact with the un-substituted polypeptide.

Modifications of this primary amino acid sequence may result in proteins which have substantially equivalent activity as compared to the unmodified counterpart polypeptide, and thus may be considered functional analogous of the parent proteins. Such modifications may be deliberate, e.g. as by site-directed mutagenesis, or they may occur spontaneous, and include splice variants, isoforms, homologues from other species, and polymorphisms. Such functional analogous are also contemplated according to the invention.

C-terminal Deletions

In another embodiment the invention provides mosquito dNK enzymes having C-terminal deletions when compared to the parent (Wild-type) enzyme. Such

deletions may be obtained by conventional techniques, e.g. site-directed mutagenesis, or as described elsvere in literature.

According to the invention it has been found that C-terminal deletions create enzymes of improved properties, in particular increased stability, improved substrate specificity, when compared to the wildtype enzyme.

In a more preferred embodiment the invention provides mosquito deoxyribonucleoside kinase enzymes having a C-terminal deletion in the order of 1-60 amino acid residues, preferably 1-50 amino acid residues, more preferred 1-40 amino acid residues, even more preferred 1-30 amino acid residues, yet more preferred 1-28 amino acid residues, most preferred 1-26 amino acid residues.

In an even more preferred embodiment, the mosquito dNK enzyme of the invention is a multifunctional deoxyribonucleoside kinase enzyme derived from *Aedes aegypti* that has a C-terminal deletion of 26 amino acid residues.

Polynucleotides Encoding Mosquito dNK

In another aspect the invention provides isolated polynucleotides encoding mosquito dNK enzymes derived from *Aedes aegypti*, preferably those mosquito dNK enzymes described above.

Hybridisation Protocol

In a preferred embodiment, the isolated polynucleotide of the invention is capable of hybridising with the polynucleotide sequence presented as SEQ ID NO: 1, or its complementary strand.

Hybridization should be accomplished under at least under at least low stringency conditions, but preferably at medium or high stringency conditions.

Suitable experimental conditions for determining hybridisation at low, medium, or high stringency conditions, respectively, between a nucleotide probe and a homologous DNA or RNA sequence, involves pre-soaking of the filter containing the DNA fragments or RNA to hybridise in 5 x SSC [Sodium chloride/Sodium citrate; cf. *Sambrook et al.*; Molecular Cloning: A Laboratory Manual, 2nd Ed., Cold Spring Harbor Lab., Cold Spring Harbor, NY 1989] for 10 minutes, and prehybridization of the filter in a solution of 5 x SSC, 5 x Denhardt's solution [cf. *Sambrook et al.; Op cit.*], 0.5% SDS and 100 µg/ml of denatured sonicated salmon sperm DNA [cf. *Sambrook et al.; Op cit.*], followed by hybridisation in the same solution containing a concentration of 10 ng/ml of a random-primed [*Feinberg A P & Vogelstein B*; Anal. Biochem. 1983 132 6-13], ^{32}P-dCTP-labeled (specific activity > 1 x 10^9 cpm/µg) probe for 12 hours at approximately 45°C.

The filter is then washed twice for 30 minutes in 2 x SSC, 0.5% SDS at a temperature of at least 55°C (low stringency conditions), more preferred of at least

60°C (medium stringency conditions), still more preferred of at least 65°C (medium/high stringency conditions), even more preferred of at least 70°C (high stringency conditions), and yet more preferred of at least 75°C (very high stringency conditions).

Molecules to which the oligonucleotide probe hybridises under these conditions may be labelled to detect hybridisation. The complementary nucleic acids or signal nucleic acids may be labelled by conventional methods known in the art to detect the presence of hybridised oligonucleotides. The most common method of detection is the use of autoradiography with e.g. ^{3}H, ^{125}I, ^{35}S, ^{14}C, or ^{32}P-labelled probes, which may then be detected using an x-ray film. Other labels include ligands, which bind to labelled antibodies, fluorophores, chemoluminescent agents, enzymes, or antibodies, which can then serve as specific binding pair members for a labelled ligand.

Identity of DNA Sequences

In another preferred embodiment, the isolated polynucleotide of the invention has at least 73%, preferably at least 75%, more preferred at least 80%, even more preferred at least 90%, yet even more preferred at least 95%, most preferred at least 98% identity to the polynucleotide sequence presented as SEQ ID NO: 1, when determined over its entire length.

In the context of this invention, "identity" is a measure of the degree of homology of nucleotide sequences. In order to characterize the identity, subject sequences are aligned so that the highest order homology (match) is obtained. Based on these general principles, the "percent identity" of two amino acid sequences or of two nucleic acids is determined using the BLASTN algorithm [*Tatiana A. Tatusova, Thomas L. Madden*: Blast 2 sequences - a new tool for comparing protein and nucleotide sequences; FEMS Microbiol. Lett. 1999 **174** 247-250], which is available from the National Center for Biotechnology Information (NCBI) web site, and using the default settings suggested here (i.e. Reward for a match = 1; Penalty for a match = -2; Strand option = both strands; Open gap = 5; Extension gap = 2; Penalties bap x_dropoff = 50; Expect = 10; Word size = 11; Filter on).

The results of this BLASTN comparison are presented in Table 2.

Table 2
BLASTN Comparison of Nucleotide Sequences of dNKs of Different Insect Origin

dNK BLASTN	*A. aegypti*	*D. melanogaster*	*B. mori*	*A. gamibiae*
A. aegypti	747/747	122/157*	--	496/664
D. melanogaster	122/157*	750/750	--	378/515
B. mori	--	--	747/747	150/213*
A. gamibiae	496/664	378/515	150/213*	741/741

Drosophila melanogaster deoxynucleoside kinase GeneBank Acc.nr.Y18048
Bombyx mori putative deoxynucleoside kinase GeneBank Acc.nr. AF226281
Anopheles gambiae deoxyribonucleoside kinase GeneBank Acc.nr. AF488801

Identities / length of the compared fragment
* similarity only to N-terminal fragment
-- No significant similarity found

Analogous DNA Sequences

In its most preferred embodiment, the isolated polynucleotide of the invention comprises the polynucleotide sequence presented as SEQ ID NO: 1 or a functional analog thereof.

In the context of this invention, the term "functional analog" covers conservatively modified polynucleotides, and polynucleotides encoding functionally equivalent polypeptides.

In the context of this invention, the term "conservatively modified polynucleotides" refers to those nucleic acids which encode identical or essentially identical amino acid sequences, or where the nucleic acid does not encode an amino acid sequence, to essentially identical sequences.

Because of the degeneracy of the genetic code, a large number of functionally identical nucleic acids encode any given protein. For instance, the codons GCA, GCC, GCG and GCU all encode the amino acid alanine. Thus, at every position where an alanine is specified by a codon, the codon can be altered to any of the corresponding codons described without altering the encoded polypeptide. Such nucleic acid variations are "silent variations," which are one species of conservatively modified variations. Every nucleic acid sequence herein, which encodes a polypeptide, also describes every possible silent variation of the nucleic acid. One of skill will recognize that each codon in a nucleic acid (except AUG, which is ordinarily the only codon for methionine) can be modified to yield a functionally identical

molecule. Accordingly, each silent variation of a nucleic acid, which encodes a polypeptide, is implicit in each described sequence.

Expression Vectors

In a third aspect the invention provides recombinant expression vectors comprising the isolated polynucleotide of the invention and a promoter operably linked to the polynucleotide.

The expression vector of the invention preferably is one suitable for carrying out expression in a eukaryotic organism.

In a more preferred embodiment the expression vector of the invention is a viral vector, in particular a *Herpes simplex* viral vector, an adenoviral vector, an adenovirus-associated viral vector, a lentivirus vector, a retroviral vector or a vacciniaviral vector.

Packaging Cell Lines

In a fourth aspect the invention provides packaging cell lines capable of producing an infective virion, which cell line comprises a vector of the invention.

Packaging cells refers to cells containing those elements necessary for production of infectious recombinant vira, which are lacking in a recombinant virus vector.

Host Cells

In a fifth aspect the invention provides an isolated host cell comprising the isolated polynucleotide of the invention, or the expression vector of the invention.

In a preferred embodiment the host cell of the invention is a eukaryotic cell, in particular a mammalian cell, a human cell, an oocyte, or a yeast cell.

In a more preferred embodiment the host cell of the invention is a human cell, a dog cell, a monkey cell, a rat cell or a mouse cell.

Pharmaceutical Compositions

In a sixth aspect the invention relates to novel pharmaceutical compositions comprising a therapeutically effective amount of the mosquito dNK enzyme of the invention, or the host cell of the invention, and a pharmaceutically acceptable carrier or diluent.

For use in therapy the mosquito deoxyribonucleoside kinase enzyme of the invention may be administered in any convenient form. In a preferred embodiment, the mosquito deoxyribonucleoside kinase enzyme of the invention is incorporated into a pharmaceutical composition together with one or more adjuvants, excipients,

carriers and/or diluents, and the pharmaceutical composition prepared by the skilled person using conventional methods known in the art.

Such pharmaceutical compositions may comprise mosquito deoxyribonucleoside kinase enzyme of the invention, or antibodies hereof. The composition may be administered alone or in combination with one or more other agents, drugs or hormones.

The pharmaceutical composition of this invention may be administered by any suitable route, including, but not limited to oral, intravenous, intramuscular, inter-arterial, intramedullary, intrathecal, intraventricular, transdermal, subcutaneous, intraperitoneal, intranasal, anteral, topical, sublingual or rectal application, buccal, vaginal, intraorbital, intracerebral, intracranial, intraspinal, intraventricular, intracisternal, intracapsular, intrapulmonary, transmucosal, or via inhalation.

Further details on techniques for formulation and administration may be found in the latest edition of Remington's Pharmaceutical Sciences (Maack Publishing Co., Easton, PA).

The active ingredient may be administered in one or several doses per day. Currently contemplated appropriate dosages are between 0.5 ng to about 50 µg/kg mosquito deoxyribonucleoside kinase/kg body weight per administration, and from about 1.0 ng/kg to about 100 µg/kg daily.

The dose administered must of course be carefully adjusted to the age, weight and condition of the individual being treated, as well as the route of administration, dosage form and regimen, and the result desired, and the exact dosage should of course be determined by the practitioner.

In further embodiments, the mosquito deoxyribonucleoside kinase of the invention may be administered by genetic delivery, using cell lines and vectors as described below under methods of treatment.

Therefore, in another preferred embodiment, the invention provides pharmaceutical compositions comprising the polynucleotide of the invention, or a vector of the invention, or a packaging cell of the invention, or a host cell of the invention, and a pharmaceutically acceptable carrier or diluent.

To generate such therapeutic cell lines, the polynucleotide of the invention may be inserted into an expression vector, e.g. a plasmid, virus or other expression vehicle, and operatively linked to expression control sequences by ligation in a way that expression of the coding sequence is achieved under conditions compatible with the expression control sequences.

Suitable expression control sequences include promoters, enhancers, transcription terminators, start codons, splicing signals for introns, and stop codons, all maintained in the correct reading frame of the polynucleotide of the invention so as

to permit proper translation of mRNA. Expression control sequences may also include additional components such as leader sequences and fusion partner sequences.

Methods of Treatment

The present invention, which relates to polynucleotides and proteins, polypeptides, peptide fragments or derivatives produced therefrom, as well as to antibodies directed against such proteins, peptides or derivatives, may be used for treating or alleviating a disorder or disease of a living animal body, including a human, which disorder or disease is responsive to the activity of a cytotoxic agent.

The disorder, disease or condition may in particular be a cancer or a viral infection.

The polynucleotides of the present invention may in particular be used as a "suicide gene", i.e. a drug-susceptibility gene. Transfer of a suicide gene to a target cell renders the cell sensitive to compounds or compositions that are relatively non-toxic to normal cells.

Therefore, in a seventh aspect, the invention provides a method for sensitising target cells to prodrugs, which method comprises the steps of

(i) transfecting the target cell with a polynucleotide sequence encoding a mosquito deoxyribonucleoside kinase enzyme that promotes the conversion of said prodrug into a (cytotoxic) drug; and

(ii) delivering said prodrug to said target cell;

wherein said target cell is more sensitive to said (cytotoxic) drug than to said prodrug.

In its broadest aspect any mosquito deoxyribonucleoside kinase enzyme may be used. However, in a preferred embodiment, the polynucleotide sequence encoding a mosquito deoxyribonucleoside kinase enzyme is a polynucleotide sequence of the invention.

In a more preferred embodiment the prodrug is a nucleoside analogue.

In the context of this invention a preferred nucleoside analogue for use according to the invention is selected from the group consisting of aciclovir (9-[2-hydroxy-ethoxy]-methyl-guanosine), buciclovir, famciclovir, ganciclovir (9-[2-hydroxy-1-(hydroxymethyl)ethoxyl-methyl]-guanosine), penciclovir, valciclovir, trifluorothymidine, AZT (3'-azido-3'-deoxythymidine), AIU (5'-iodo-5'-amino-2',5'-dideoxyuridine), ara-A (adenosine-arabinoside; Vivarabine), ara-C (cytidine-arabinoside), ara-G (9-beta-D-arabinofuranosylguanine), ara-T, 1-beta-D-arabinofuranosyl thymine, 5-ethyl-2'-deoxyuridine, 5-iodo-5'-amino-2,5'-dideoxyuridine, 1-[2-deoxy-2-fluoro-beta-D-arabino furanosyl]-5-iodouracil, idoxuridine (5-iodo-2'deoxyuridine), fludarabine (2-Fluoroadenine 9-beta-D-Arabinofuranoside), 3'-deoxyadenosine (3-dA), 2`,3`-dideoxyinosine (ddI), 2`,3`-dideoxycytidine (ddC), 2`,3`-dideoxythymidine (ddT), 2`,3`-

dideoxyadenosine (ddA), 2`,3`-dideoxyguanosine (ddG), 2-chloro-2`-deoxyadenosine (2CdA), 5-fluorodeoxyuridine, BVaraU ((E)-5-(2-bromovinyl)-1-beta-D-arabinofuranosyluracil), BVDU (5-bromovinyl-deoxyuridine), FIAU (1-(2-deoxy-2-fluoro-beta-D-arabinofuranosyl)-5-iodouracil), 3TC (2`-deoxy-3`-thiacytidine), dFdC gemcitabine (2`,2`-difluorodeoxycytidine), dFdG (2`,2`-difluorodeoxyguanosine), 5-fluorodeoxyuridine (FdUrd), d4T (2`,3`didehydro-3`-deoxythymidine), ara-M (6-methoxy purinearabinonucleoside), IudR (5-Jodo-2`deoxyuridine), clofarabine (chloro-2`-fluoro-deoxy-9-beta-D-arabinofuranosyladenine), CaFdA (2-chloro-2-ara-fluoro-deoxyadenosine), ara-U (1-beta-D-arabinofuranosyluracil), FBVAU (E)-5-(2-bromovinyl)-1-(2-deoxy-2-fluoro-beta-D-arabinofuranosyl)uracil, FMAU 1-(2-deoxzy-2-fluoro-beta-D-arabinofuranosyl)-5-methyluracil, FLT 3`-fluoro-2`-deoxythymidine, 5-Br-dUrd 5-bromodeoxyuridine, 5-Cl-dUrd 5-chlorodeoxyuridine or dFdU 2`,2`-difluorodeoxyuridine.

In a more preferred embodiment the nucleoside analog for use according to the invention is gemcitabine (dFdC, 2`,2`-difluorodeoxycytidine),

The dNK enzyme invention may be used directly via e.g., injected, imparaziteed or ingested pharmaceutical compositions to treat a pathological process responsive to the mosquito deoxyribonucleoside kinase enzyme.

The polynucleotide of the invention, including the complementary sequences thereof, may be used for the expression of the dNK kinase enzyme of the invention. This may be achieved by cell lines expressing such proteins, peptides or derivatives of the invention, or by virus vectors encoding such proteins, peptides or derivatives of the invention, or by host cells expressing such proteins, peptides or derivatives. These cells, vectors and compositions may be administered to treatment target areas to affect a disease process responsive to cytotoxic agents.

Suitable expression vectors may be a viral vector derived from *Herpes simplex*, adenovira, lentivira, retrovira, or vaccinia vira, or from various bacterially produced plasmids, and may be used for *in vivo* delivery of nucleotide sequences to a whole organism or a target organ, tissue or cell population. Other methods include, but are not limited to, liposome transfection, electroporation, transfection with carrier peptides containing nuclear or other localising signals, and gene delivery via slow-release systems. In still another aspect of the invention, "antisense" nucleotide sequences complementary to the nucleotide of the invention or portions thereof, may be used to inhibit or enhance mosquito deoxyribonucleoside kinase enzyme expression.

In another preferred embodiment the invention provides methods for inhibiting pathogenic agents in warm-blooded animals, which methods comprises the step of administering to said animal a polynucleotide of the invention, or an expression vector of the invention.

In a more preferred embodiment the polynucleotide sequence or the expression vector is administered *in vivo*.

In another preferred embodiment the pathogenic agent is a virus, a bacteria or a parasite, or even a tumour cell.

In another preferred embodiment the pathogenic agent is an autoreactive immune cell.

In an even more preferred embodiment the method further comprises the step of administering a nucleoside analogue to said warm-blooded animal.

Preferably the nucleoside analogue is selected from those described above.

In a most preferred embodiment the nucleoside analog for use according to the invention is gemcitabine (2`,2`-difluorodeoxycytidine).

Imaging

Suicide gene therapy, i.e. transfection of a so-called suicide gene that sensitizes target cells towards a prodrug, offers an attractive approach for treating malignant tumors. For the development of effective clinical suicide gene therapy protocols, a non-invasive method to assay the extent, the kinetics and the spatial distribution of transgene expression is essential. Such imaging methods allow investigators and physicians to assess the efficiency of experimental and therapeutic gene transfection protocols and would enable early prognosis of therapy outcome.

Radionuclide imaging techniques like single photon emission computed tomography (SPECT) and positron emission tomography (PET), which can non-invasively visualize and quantify metabolic processes in vivo, are being evaluated for repetitive monitoring of transgene expression in living animals and humans. Transgene expression can be monitored directly by imaging the expression of the therapeutic gene itself, or indirectly using a reporter gene that is coupled to the therapeutic gene. Various radiopharmaceuticals have been developed and are now being evaluated for imaging of transgene expression.

Therefore, in another aspect, the invention provides a method of non-invasive nuclear imaging of transgene expression of a plant deoxynucleoside kinase enzyme of the invention in a cell or subject, which method comprises the steps of

(i) transfecting said cell or subject with a polynucleotide sequence encoding the plant deoxynucleoside kinase enzyme, which enzyme promotes the conversion of a substrate into a substrate-monophosphate;

(ii) delivering said substrate to said cell or subject; and

(iii) non-invasively monitoring the change to said prodrug in said cell or subject.

In a preferred embodiment the monitoring carried out in step (iii) is performed by Single Photon Emission Computed Tomography (SPECT), by Positron Emission Tomography (PET), by Magnetic Resonance Spectroscopy (MRS), by Magnetic Resonance Imaging (MRI), or by Computed Axial X-ray Tomography (CAT), or a combination thereof

In a more preferred embodiment the substrate is a labelled nucleoside analogue selected from those listed above. The labelled nucleoside analogue preferably contains at least one radionuclide as a label. Positron emitting radionuclides are all candidates for usage. In the context of this invention the radionuclide is preferably selected from ^{2}H (deuterium), ^{3}H (tritium), ^{11}C, ^{13}C, ^{14}C, ^{15}O, ^{13}N, ^{123}I, ^{125}I, ^{131}I, ^{18}F and ^{99m}Tc.

An example of commercially available labelling agents, which can be used in the preparation of the labelled nucleoside analogue is $[^{11}C]O_2$, ^{18}F, and NaI with different isotopes of Iodine. In particular $[^{11}C]O_2$ may be converted to a $[^{11}C]$-methylating agent, such as $[^{11}C]H_3I$ or $[^{11}C]$-methyl triflate.

Method of Phosphorylating Nucleosides

The mosquito deoxyribonucleoside kinase enzyme of the invention may find different utility, including both therapeutic and biotechnological applications.

In an eight aspect the invention relates to use of the mosquito deoxyribonucleoside kinase enzyme of the invention for phosphorylating nucleosides or a nucleoside analogs.

In a preferred embodiment the invention provides a method for phosphorylating a nucleoside or a nucleoside analog, comprising the steps of

i) subjecting the nucleoside or nucleoside analog to the action of the mosquito deoxyribonucleoside kinase enzyme of the invention; and

ii) recovering the phosphorylated nucleoside or nucleoside analog.

EXAMPLES

The invention is further illustrated with reference to the following examples, which are not intended to be in any way limiting to the scope of the invention as claimed.

Example 1

Cloning of *Aedes aegypti* dNK

This example describes how the gene encoding the Aedes aegypti dNK kinase of the invention was identified, and how vector to express dNK kinase was constructed.

The expressed sequence tag library of the GeneBank database at the National Institute for Biotechnology Information (http://www.ncbi.nlm.nih.gov/) was searched with the Translated BLAST search Tool (Protein query – Translated db, TBLASTN) to identify cDNA clones that encode enzymes similar to Drosophila melanogaster dNK. An EST clone deposited by Dr. Guiyun Yan (Department of Biological Sciences, State University of New York at Buffalo) was identified and obtained for the same source. A plasmid comprising the expressed sequence tag inserted in the vector pBK-CMV (ZAP Express Vector, Stratagene) was fully sequenced using the plasmid specific T7 and T3 primers. The DNA sequence determination revealed ORF of 747 bp (SEQ.ID.NO: 1) which encode a protein of 248 amino acid residues (SEQ.ID.NO: 2). The calculated molecular mass of the protein was 28792 Da with 7.18 pI. The greatest similarity of the protein was to *Anopheles gamibiae* dNK (78% identities (191/244) and 88% similarities (215/244), no gaps) and *Drosophila melanogaster* dNK (64% identities (159/248), 76% simlarity (189/248) and 1% gaps (4/248)).

Aedes aegypti dNK Kinase

To obtain C terminus GST tagged version, the full ORF of the mosquito dNK kinase was amplified by PCR using the cloning primers which were designed based on the newly obtained sequence data. The following primers were used:

5' TTAGGATCCATGGCGGCTGCCATCGGAC 3' (SEQ.ID.NO: 3) and
5' CAGCAATTGTTAGAATTCAGTTCTCGATCG 3' (SEQ.ID.NO: 4)

The PCR fragment was subsequently cut by BamHI/MfeI and ligated into pGEX-2T vector (Amersham-Pharmacia), which was precut with EcoRI/BamHI. The resulting plasmid was named PZG318.

HSV1 thymidine kinase (used for control)

The thymidine kinase from HSV1 was amplified using the primers
5' CGCGGATCCATGGCTTCGTACCCCGGCCATC 3' (HSV-for A; SEQ ID NO: 5); and
5' CCGGAATTCTTAGTTAGCCTCCCCCATCTCCCG 3' (HSV-rev; SEQ ID NO: 6);

and using the plasmid pCMV-pacTK described by *Karreman* [*Christiaan Karreman*; Gene 1998 **218** 57-62] as template.

The PCR fragment was subsequently cut by *EcoRI/BamHI* and ligated into pGEX-2T vector (Amersham-Pharmacia) that was also cut by *EcoRI/BamHI*.

The resulting plasmid was named pGEX-2T-HSV-TK.

Example 2

Expression and dNK activity

This example describes how *E. coli* KY895 were transformed with the plasmid obtained according to Example 1, in order to express mosquito dNK.

KY895 cells were transformed by the expression plasmid of Example 1 using standard techniques, e.g. as described by e.g. *Sambrook et al.* [*Sambrook et al.*; Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Lab., Cold Spring Harbor, NY 1989].

Transformed cells were grown to an OD600nm of 0.5-0.6 in LB/Ampicillin (100 µg/ml) medium at 37°C and protein expression was induced by addition of 100 µM IPTG. The cells were further grown for 4 h at 25°C and subsequently harvested by centrifugation. The cell pellet was subjected to sonification in the binding buffer A (20 mM $NaPO_4$ pH 7,3; 150 mM NaCl; 10% Glycerol; and 0.1% Triton X-100) and subjected to centrifugation at 10,000 x g for 30 minutes. Cell free extract was used for enzymatic activity assays.

Nucleoside kinase activities were determined by initial velocity measurments based on four time sample by the DE-81 filter paper assay using tritium labelled substrates. The assays were performed as described by *Munch-Petersen et al.* [*Munch-Petersen B, Knecht W, Lenz C, Søndergaard L, Piškur J*: Functional expression of a multisubstrate deoxyribonucleoside kinase from Drosophila melanogaster and its C-terminal deletion mutants; J. Biol. Chem. 2000 **275** 6673-6679].

The protein concentration was determined according to *Bradford* with BSA as standard protein [*Bradford M M:* A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding; Anal. Biochem. 1976 **72** 248-254]. SDS-PAGE was done according to the procedure of *Laemmli* [*Laemmli U K*: Cleavage of structural proteins during the assembly of the head of bacteriophage T4; Nature 1970 **227** 680-685], and proteins were visualized by Coomassie staining to verify recombinant protein expression.

Kinetic data were evaluated by nonlinear regression analysis using the Michaelis-Menten equation $v = V_{max} \times [S]/(K_m + [S])$ or Hill-equation $v = V_{max} \times [S]^h/(K_{0.5}^h + [S]^h)$ as described by *Knecht et al.* [*Knecht W, Bergjohann U, Gonski S, Kirschbaum B & Löffler M*: Functional expression of a fragment of human

dihydroorotate dehydrogenase by means of the baculovirus expression vector system and kinetic investigation of the purified recombinant enzyme; Eur. J. Biochem. 1996 240 292-301].

The natural deoxyribonucleoside and deoxyribonucleoside analogs were tested at a fixed concentration of 100 µM. The specific activity in each extract is given in mU/ml.

The results of these evaluations are presented in Table 3 and 4.

Table 3

Mosquito Deoxyribonucleoside Kinase Activity in Extracts of KY895 Cells

KY895 transformed with	Thd	dCyt	aAdo	dGuo
cells only	n.d.	n.d.	n.d.	n.d.
pGEX-2T	n.d.	0.1	n.d.	n.d.
PZG318	125	124.5	364	214.7

The deoxyribonucleoside kinases from *Aedes aegypti* (PZG318) was able to phosphorylate all four deoxynucleosides namely dThd, Cyd, dAdo and dGuo. This shows that the mosquito deoxyribonucleoside kinase is clearly multisubstrate kinase.

Table 4

Mosquito Deoxyribonucleoside Kinase Activity with analogs in Extracts of KY895 Cells

KY895 transformed with	ACV	GCV	dFdC
cells only	n.d.	n.d.	n.d.
pGEX-2T	n.d.	n.d.	n.d.
PZG318	0.3	0.7	228

n.d. stands for not detectable

The data in this table show that mosquito enzyme activates gemcitabine (dCyt nucleoside analog) much stronger than dCyt which is natural substrate for this kinase (see table 3). In addition ACV and GCV are also activated.

Example 3

Growth of Transformed *E. coli* KY895 on Nucleoside Analog Plates

This example describes how host cells transformed with the plasmids obtained according to Example 1 are able to grow on plates in presence of the

nucleoside analog gemcitabine (dFdC, 2',2'-difluorodeoxycytidine) and ara-C (cytidine-arabinoside)

Deoxyribonucleoside kinases are of interest as suicide-genes to be used in gene-mediated therapy of cancer or viral infections. In this example the potential of the mosquito kinase of the invention to convert different nucleoside analogs are compared to that of the human *Herpes simplex* virus 1 thymidine kinase (HSV1-TK) in a bacterial test system.

The experiment was carried out essentially as described by *Knecht et al.* [*Knecht W, Munch-Petersen B and Piškur J*: Identification of residues involved in the specificity and regulation of the highly efficient multisubstrate deoxyribonucleoside kinase from Drosophila melanogaster; J. Mol. Biol. 1970 **301** 827-837]. Briefly, overnight cultures of transformed KY895 were diluted 200-fold in 10% glycerol and 2 µl drops of the dilutions were spotted on M9 minimal medium plates [*Ausubel F, Brent R, Kingston R E, Moore D D, Seidman J G, Smith J A & Struhl K* (Eds.): Short protocols in molecular biology; 3rd edition (1995) pp.1-2, Wiley, USA] supplemented with 0.2% glucose, 0.1% casamino acids, 100 µg/ml ampicillin and with or without nucleoside analogs. Growth was inspected visually after 24 hours of incubation at 37°C.

The results of the experiment are presented in Table 4 below.

Table 5

Growth of KY895 in presence of gemcitabine and araC

KY895 transformed with	dFdC LD_{100} (nM)	ara-C LD_{100} (µM)
cells only	>100	> 1000
pGEX-2T	>100	>1000
PZG318	3.16	100
pGEX-2T-HSV1-TK	>100	>1000

pGEX-2T is the vector and is available from Amersham-Pharmacia;

As can be seen from Table, mosquito dNK kinase (PZG318) was very efficient, as reflected by the lowest LD_{100}, in killing KY895 on dFdC and araC plates. The LD_{100} for dFdC was 30-fold and for ara-C 10-fold lower than that of HSV1-TK, that sensitised the cells to the same degree as the empty plasmid pGEX-2T.

CLAIMS

1. An isolated polynucleotide encoding a mosquito deoxyribonucleoside kinase derived from a yellow fever mosquito, said isolated polynucleotide being selected from the group consisting of:

i) an isolated polynucleotide encoding multisubstrate deoxyribonucleoside kinase derived from yellow fever mosquito *Aedes aegypti*,

ii) an isolated polynucleotide encoding a deoxyribonuleoside kinase enzyme derived from a *Aedes aegypti*, which kinase enzyme, when compared to human *Herpes simplex* virus 1 (HSV-TK1) and upon transformation into an eukaryotic cell, decreases at least four (4) fold the IC_{50} of at least one nucleoside analogue,

iii) an isolated polynucleotide encoding a deoxyribonuleoside kinase enzyme derived from a *Aedes aegypti*, which kinase enzyme, when expressed and compared to human *Herpes simplex* virus 1 (HSV-TK1), has a decreased ratio of [kcat/km (dCyt)] / [kcat/km (dFdC)] of at least two (2) fold,

iv) an isolated mutated and/or truncated polynucleotide encoding a deoxyribonucleoside kinase variant derived from mosquito, which deoxyribonucleoside kinase enzyme variant, when compared to human *Herpes simplex* virus 1 (HSV-TK1) and upon transformation into an eukaryotic cell, decreases at least four (4) fold the IC_{50} of at least one nucleoside analogue.

2. The polynucleotide of claim 1, encoding a deoxyribonuleoside kinase enzyme derived from *Aedes aegypti*.

3. The isolated polynucleotide of claim 1, which is capable of hybridising under at least medium stringency conditions with the polynucleotide sequence presented as SEQ ID NO: 1, or its complementary strand.

4. The isolated polynucleotide of claim 1, which has at least 73%, preferably at least 75%, more preferred at least 80%, even more preferred at least 90%, yet more preferred at least 95%, most preferred at least 98% identity to the polynucleotide sequence presented as SEQ ID NO: 1 when determined over its entire length.

5. The isolated polynucleotide of any of claims 1-4, comprising the polynucleotide sequence presented as SEQ ID NO: 1, or a functional analog thereof.

6. The isolated mutated and/or truncated polynucleotide of claim 1, which deoxyribonuleoside kinase enzyme variant, when compared to human *Herpes simplex*

virus 1 (HSV-TK1) and upon transformation into a eukaryotic cell, decreases at least two (2) fold the IC_{50} of dCyt.

7. An isolated mosquito deoxyribonucleoside kinase enzyme selected from the group consisting of:

i) an isolated mosquito deoxyribonucleoside kinase enzyme encoded by the polynucleotide of any one of the claims 1-6,

ii) an isolated mosquito deoxyribonucleoside kinase enzyme derived from from yellow fever mosquito *Aedes aegypti*,

iii) an isolated mosquito multisubstrate deoxyribonucleoside kinase enzyme, which mosquito multisubstrate deoxyribonucleoside kinase enzyme, when compared to human *Herpes simplex* virus 1 (HSV-TK1) in an eukaryotic cell, decreases at least four (4) fold the IC_{50} of at least one nucleoside analogue

iv) an isolated mutated and/or truncated multisubstrate deoxyribonucleoside kinase enzyme derived from a mosquito, which multisubstrate deoxyribonucleoside kinase enzyme, when expressed and compared to human *Herpes simplex* virus 1 (HSV-TK1) and upon transduction into a eukaryotic cell, decreases at least four (4) fold the IC_{50} of at least one nucleoside analogue.

8. The isolated multisubstrate deoxyribonucleoside kinase of claim 7, being derived from yellow fever mosquito *Aedes aegypti*.

9. The mosquito deoxyribonuleoside kinase of claim 7 comprising the amino acid sequence of SEQ ID NO: 2, or an amino acid sequence of at least 70%, preferably at least 80%, even more preferred at least 90%, yet more preferred at least 95%, most preferred at least 98% identity with this sequence, when determined over its entire length.

10. The mosquito deoxyribonuleoside kinase of claims 7 or 8 comprising the amino acid sequence of SEQ ID NO: 2, or a functional analogue thereof.

11. The mosquito deoxyribonuleoside kinase of any one of claims 7-10, which decreases at least three (3) fold the lethal dose (LD_{100}) of at least one nucleoside analogue when compared to the action of a thymidine kinase derived from human *Herpes simplex* virus 1 (HSV-TK1).

12. A vector construct comprising the polynucleotide of any of claims 1-6, and a promoter operably linked to the polynucleotide.

13. The vector construct of claim 12 being a viral vector, in particular a *Herpes simplex* viral vector, an adenoviral vector, an adenovirus-associated viral vector, a lentivirus vector, a retroviral vector or a vacciniaviral vector.

14. A packaging cell line capable of producing an infective virion comprising the vector of either of claims 12-13.

15. A host cell comprising the polynucleotide of any of claims 1-6, or the vector of either of claims 12-13.

16. The host cell of claim 15, which is a human cell, a dog cell, a monkey cell, a rat cell or a mouse cell.

17. A pharmaceutical composition comprising the mosquito deoxyribonucleoside kinase enzyme of any of claims 7-11, and a pharmaceutically acceptable carrier or diluent.

18. A pharmaceutical composition comprising the polynucleotide of any of claims 1-6, or a vector of either of claims 12-13, and a pharmaceutically acceptable carrier or diluent.

19. A pharmaceutical composition comprising the packaging cell line of claim 14, or the host cell of either of claims 15-16, and a pharmaceutically acceptable carrier or diluent.

20. A method of sensitising a cell to a prodrug, which method comprises the steps of

(i) transfecting said cell with a polynucleotide sequence encoding a mosquito deoxyribonucleoside kinase enzyme that promotes the conversion of said prodrug into a (cytotoxic) drug; and

(ii) delivering said prodrug to said cell;

wherein said cell is more sensitive to said (cytotoxic) drug than to said prodrug.

21. The method of claim 20, wherein the polynucleotide sequence encoding a mosquito deoxyribonucleoside kinase enzyme is a polynucleotide sequence of any of claims 1-6.

22. The method of either of claims 20-21, wherein the prodrug is a nucleoside analogue.

23. The method of claim 22, wherein the nucleoside analogue is gemcitabine (dFdC).

24. A method of inhibiting a pathogenic agent in a warm-blooded animal, which method comprises administering to said animal a polynucleotide of any of claims 1-6, or a vector of either of claims 12-13.

25. The method of claim 24, wherein said polynucleotide sequence or said vector is administered *in vivo*.

26. The method of either of claims 24-25, wherein said pathogenic agent is a virus, a bacteria or a parasite.

27. The method of either of claims 24-25, wherein said pathogenic agent is a tumour cell.

28. The method of either of claims 24-25, wherein said pathogenic agent is an autoreactive immune cell.

29. The method of any of claims 24-28, further comprising the step of administering a nucleoside analogue to said warm-blooded animal.

30. The method of claim 29, wherein said nucleoside analogue is gemcitabine (dFdC).

31. Use of the mosquito deoxyribonucleoside kinase enzyme of any of claims 7-11 for the phosphorylation of a nucleoside or a nucleoside analog.

32. A method of phosphorylating a nucleoside or a nucleoside analog, comprising the steps of

i) subjecting the nucleoside or nucleoside analog to the action of the mosquito deoxyribonucleoside kinase enzyme of any of claims 7-11, and

ii) recovering the phosphorylated nucleoside or nucleoside analog.

33. A method of non-invasive nuclear imaging of transgene expression of a mosquito deoxyribonucleoside kinase enzyme of the invention in a cell or subject, which method comprises the steps of

(i) transfecting said cell or subject with a polynucleotide sequence encoding the mosquito deoxyribonucleoside kinase enzyme, which enzyme promotes the conversion of a substrate into a substrate-monophosphate;
(ii) delivering said substrate to said cell or subject; and
(iii) non-invasively monitoring the change to said prodrug in said cell or subject.

34. The method of claim 33, wherein the monitoring carried out in step (iii) is performed Single Photon Emission Computed Tomography (SPECT), by Positron Emission Tomography (PET), by Magnetic Resonance Spectroscopy (MRS), by Magnetic Resonance Imaging (MRI), or by Computed Axial X-ray Tomography (CAT), or a combination thereof.

35. The method of either of claims 33-34, wherein the substrate is a labelled nucleoside analogue.

ABSTRACT

YELLOW FEVER MOSQUITO DEOXYRIBONUCLEOSIDE KINASES AND ITS USE

This invention relates to mosquito multisubstrate deoxyribonucleoside kinases and their use in gene therapy. More specifically the invention provides novel deoxyribonucleoside kinases derived from *Aedes aegypti*.

In further aspects the invention provides novel polynucleotides encoding the mosqiuto deoxyribonucleoside kinases, vector constructs comprising the polynucleotide, host cells carrying the polynucleotide or vector, methods of sensitising cells to prodrugs, method of inhibiting pathogenic agents in warm-blooded animals, methods of synthesizing monophosphates, imagining applications and pharmaceutical compositions comprising the mosquito deoxyribonucleoside kinases of the invention.

SEQUENCE LISTING

Aedes aegypti dNK

SEQ ID NO: 1

```
atg gcg gct gcc atc gga ccg gag cgg ctt ggc gtg gcc gga aag aag      48
Met Ala Ala Ala Ile Gly Pro Glu Arg Leu Gly Val Ala Gly Lys Lys
 1               5                  10                  15

ccc ttc act gtt ttc att gag gga aac atc ggc agc gga aag acc aca      96
Pro Phe Thr Val Phe Ile Glu Gly Asn Ile Gly Ser Gly Lys Thr Thr
            20                  25                  30

ttc ctg aac cac ttc gag aaa ttc aag gat agg gtt tgt ctg ctg acg     144
Phe Leu Asn His Phe Glu Lys Phe Lys Asp Arg Val Cys Leu Leu Thr
        35                  40                  45

gaa cct gtg gaa aag tgg cgg gac tgc ggg gga gtc aat ctg ctg gat     192
Glu Pro Val Glu Lys Trp Arg Asp Cys Gly Gly Val Asn Leu Leu Asp
    50                  55                  60

cta atg tac aag gaa ccg cac cgt tgg gcg atg ccg ttc cag acc tac     240
Leu Met Tyr Lys Glu Pro His Arg Trp Ala Met Pro Phe Gln Thr Tyr
65                  70                  75                  80

gtt act ttg acg atg ctc aac atg cat act tat caa acg gat aaa agc     288
Val Thr Leu Thr Met Leu Asn Met His Thr Tyr Gln Thr Asp Lys Ser
                85                  90                  95

gtg aag ctc atg gaa cgg tcc atg ttc agt gcc aga tat tgt ttc gtg     336
Val Lys Leu Met Glu Arg Ser Met Phe Ser Ala Arg Tyr Cys Phe Val
            100                 105                 110

gaa aac atg ctc gcg tct ggt agc tta cat cag gga atg tac aat att     384
Glu Asn Met Leu Ala Ser Gly Ser Leu His Gln Gly Met Tyr Asn Ile
        115                 120                 125

cta caa gag tgg tat gag ttc atc cat gcc aat ata cac att caa gca     432
Leu Gln Glu Trp Tyr Glu Phe Ile His Ala Asn Ile His Ile Gln Ala
    130                 135                 140

gat ttg ata gtt tat tta cga act agt ccg gaa atc gtt tat gag cga     480
Asp Leu Ile Val Tyr Leu Arg Thr Ser Pro Glu Ile Val Tyr Glu Arg
145                 150                 155                 160

atg aaa aag cgc gca aga tcg gaa gaa agt tgc gtt ccg tta aaa tat     528
Met Lys Lys Arg Ala Arg Ser Glu Glu Ser Cys Val Pro Leu Lys Tyr
                165                 170                 175

cta caa gaa cta cac gag ctg cat gaa aac tgg cta atc cac gga act     576
Leu Gln Glu Leu His Glu Leu His Glu Asn Trp Leu Ile His Gly Thr
            180                 185                 190

ttc ccg aga gta gcc ccg gtc ctc gtt ttg gat gca gac tta gac ttg     624
Phe Pro Arg Val Ala Pro Val Leu Val Leu Asp Ala Asp Leu Asp Leu
        195                 200                 205

cac aac atc agc tca gaa tac aag cga tcc gaa acc agc att ctc aag     672
His Asn Ile Ser Ser Glu Tyr Lys Arg Ser Glu Thr Ser Ile Leu Lys
    210                 215                 220

cct att ctc ata gat aat acc aac cag cat ccc att ctt gca tca ccc     720
Pro Ile Leu Ile Asp Asn Thr Asn Gln His Pro Ile Leu Ala Ser Pro
225                 230                 235                 240

agc aaa cga tcg aga act gaa ttc taa                                 747
Ser Lys Arg Ser Arg Thr Glu Phe
                245
```

Aedes aegypti protein seq

SEQ ID NO: 2

```
Met Ala Ala Ala Ile Gly Pro Glu Arg Leu Gly Val Ala Gly Lys Lys
 1               5                  10                  15

Pro Phe Thr Val Phe Ile Glu Gly Asn Ile Gly Ser Gly Lys Thr Thr
            20                  25                  30

Phe Leu Asn His Phe Glu Lys Phe Lys Asp Arg Val Cys Leu Leu Thr
        35                  40                  45

Glu Pro Val Glu Lys Trp Arg Asp Cys Gly Gly Val Asn Leu Leu Asp
    50                  55                  60

Leu Met Tyr Lys Glu Pro His Arg Trp Ala Met Pro Phe Gln Thr Tyr
65                  70                  75                  80

Val Thr Leu Thr Met Leu Asn Met His Thr Tyr Gln Thr Asp Lys Ser
                85                  90                  95

Val Lys Leu Met Glu Arg Ser Met Phe Ser Ala Arg Tyr Cys Phe Val
            100                 105                 110

Glu Asn Met Leu Ala Ser Gly Ser Leu His Gln Gly Met Tyr Asn Ile
        115                 120                 125

Leu Gln Glu Trp Tyr Glu Phe Ile His Ala Asn Ile His Ile Gln Ala
    130                 135                 140

Asp Leu Ile Val Tyr Leu Arg Thr Ser Pro Glu Ile Val Tyr Glu Arg
145                 150                 155                 160

Met Lys Lys Arg Ala Arg Ser Glu Glu Ser Cys Val Pro Leu Lys Tyr
                165                 170                 175

Leu Gln Glu Leu His Glu Leu His Glu Asn Trp Leu Ile His Gly Thr
            180                 185                 190

Phe Pro Arg Val Ala Pro Val Leu Val Leu Asp Ala Asp Leu Asp Leu
        195                 200                 205

His Asn Ile Ser Ser Glu Tyr Lys Arg Ser Glu Thr Ser Ile Leu Lys
    210                 215                 220

Pro Ile Leu Ile Asp Asn Thr Asn Gln His Pro Ile Leu Ala Ser Pro
225                 230                 235                 240

Ser Lys Arg Ser Arg Thr Glu Phe
                245
```

Description of Artificial Sequence: PCR primer sequence

SEQ ID NO: 3

TTA GGA TCC ATG GCG GCT GCC ATC GGA C

SEQ ID NO: 4

CAG CAA TTG TTA GAA TTC AGT TCT CGA TCG

SEQ ID NO: 5

CGC GGA TCC ATG GCT TCG TAC CCC GGC CAT C

SEQ ID NO: 6

CCG GAA TTC TTA GTT AGC CTC CCC CAT CTC CCG